Microbial transformation of some steroids by *Cladosporium cladosporioides* MRC 70282

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Incubation of testosterone, androstenedione and progesterone with *Cladosporium cladosporioides* MRC 70282 for 5 days is reported. Testosterone was hydroxylated at C-16β and then oxidised at C-16. This was accompanied by a minor independent oxidation at C-17 and epimerisation at C-17. Androstenedione was reduced at C-17 and hydroxylated at C-16β, and this was then oxidised to a ketone at C-16. This was accompanied by a minor epimerisation at C-17. Progesterone was hydroxylated at C-21, and this was accompanied by a minor independent 5α-reduction.

Keywords: testosterone, androstenedione, progesterone, *Cladosporium cladosporioides*, biotransformation

Steroids are biologically and pharmaceutically important substances that have many physiological activities. There are more than 300 approved steroid-based drugs that are widely used for medicinal purposes that have anti-inflammatory, antimicrobial, antitumour, antioestrogenic, antiallergenic, antidiabetic, anti-HIV and anti-convulsant activity.[1]

The production of steroids and their derivatives by conventional synthetic routes has some disadvantages, and these can be time-consuming, expensive and environmentally unfriendly processes. In contrast, microbial steroid biotransformations have overcome these disadvantages and are widely used to produce steroid hormones and drugs because of their high regio- and stereoselectivities.[1,2]

Cladosporium is a large genus of the Ascomyta. *Cladosporium* species are mostly saprophytes and also include common endophytes and plant pathogens, and they are even hyperparasites of other fungi. Some *Cladosporium* species are also considered pathogenic to humans and animals.[3]

Cladosporium cladosporioides is an endophytic fungus that is widely distributed globally. This fungus is pathogenic to some plants and is also considered pathogenic to animals and humans.[3,4] In this work, testosterone **1**, androstenedione **2** and progesterone **3** were incubated with *C. cladosporioides* MRC 70282 for 5 days in order to investigate their metabolism.

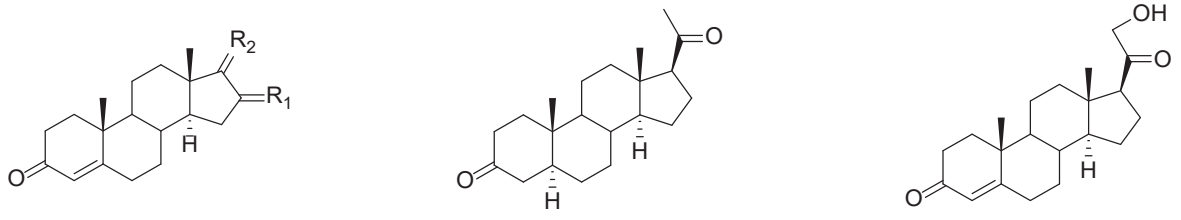
Incubation of testosterone **1** with *C. cladosporioides* MRC 70282 for 5 days yielded four metabolites (Table 1). The first metabolite was identified as androstenedione **2** (Fig. 1). The ^{13}C NMR spectrum of **2** lacked the C-17 resonance of **1** at δ_C 80.75 ppm and showed a new resonance at δ_C 220.43 ppm, indicating an oxidation at C-17. The absence of the characteristic 17α-H resonance (1H, t, J = 8.5 Hz) of **1** at δ_H 3.65 ppm further indicated that an oxidation at C-17 had occurred. The NMR data of **2** were comparable with the literature.[5]

The second metabolite was identified as 17β-hydroxyandrost-4-ene-3,16-dione **4**. The ^{13}C NMR spectrum of **4** had a new carbon atom resonance at δ_C 216.61 ppm, indicating the presence of a carbonyl group in ring D. The metabolite retained the 17α-H resonance of **1** as a broad singlet at δ_H 3.77 ppm, suggesting that an oxidation had taken place at C-16. In the ^{13}C NMR spectrum of **4** there was a significant downfield shift (Δ 5.29 ppm) for the C-17 resonance, suggesting that a hydroxylation had taken place at C-16 and the resulting alcohol had been subsequently oxidised to a ketone. The NMR data of **4** were consistent with the literature.[6]

The third metabolite was identified as 16β,17β-dihydroxyandrost-4-ene-3-one **5**. The ^{13}C NMR spectra of **5** contained a resonance at δ_C 80.48 ppm, suggesting that the

Table 1 Yields of metabolite following chromatography

Substrate	Metabolite	% Yield
Testosterone **1**		
	Androstenedione **2**	8
	17β-Hydroxyandrost-4-ene-3,16-dione **4**	31
	16β,17β-Dihydroxyandrost-4-ene-3-one **5**	20
	16β,17α-Dihydroxyandrost-4-ene-3-one **6**	2
Androstenedione **2**		
	Testosterone **1**	2
	17β-Hydroxyandrost-4-ene-3,16-dione **4**	30
	16β,17β-Dihydroxyandrost-4-ene-3-one **5**	24
	16β,17α-Dihydroxyandrost-4-ene-3-one **6**	2
Progesterone **3**		
	5α-Pregnane-3,20-dione **7**	12
	21-Hydroxypregn-4-ene-3,20-dione **8**	40



1 R_1=H_2, R_2=βOH, αH
2 R_1=H_2, R_2=O
3 R_1=H_2, R_2=β$COCH_3$, αH
4 R_1=O, R_2=βOH, αH
5 R_1=βOH, αH, R_2=βOH, αH
6 R_1=βOH, αH, R_2=βH, αOH

7 **8**

Fig. 1 Structures of steroidal substrates and their metabolites.

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17β-hydroxyl group of **1** had been retained. The metabolite also showed new resonances at δ_H 4.15 ppm (1H, td, J = 7.5 and 5.0 Hz) and δ_C 69.74 ppm, indicating the presence of a new hydroxyl group. The characteristic triplet of 17α-H now appeared as a doublet due to coupling with a proton at C-16. The ^{13}C NMR spectrum of **5** showed a downfield shift for C-15 (Δ 11.94 ppm), whereas there was a γ-gauche upfield shift for C-14 (Δ 3.12 ppm), further indicating the presence of a hydroxyl group at C-16. When compared to the literature, the change in the characteristic multiplicity of 17α-H resonance suggested the presence of a 16β-*hydroxy*l group.[5] The NMR data and melting point of **5** were comparable with the literature.[5,6]

The fourth metabolite was identified as 16β,17α-dihydroxyandrost-4-ene-3-one **6**. The ^{13}C NMR-spectrum of **6** had new resonances at δ_C 86.54 ppm and δ_C 80.83 ppm, which were assigned to two hydroxyl groups. The resonance (1H, s) at δ_H 3.60 ppm was assigned to a single proton attached to C-17. This is normally a characteristic triplet for 17α-H or doublet for 17β-H.[7] The change in the multiplicity of this resonance suggested that the other hydroxyl group was at C-16. The ^{13}C NMR spectrum of **6** showed a downfield shift for C-15 (Δ 12.33 ppm), whereas it showed a small γ-gauche upfield shift for C-14 (Δ 1.84 ppm), further suggesting that the other hydroxyl group was at C-16. When compared to the literature, the multiplicities of the resonances at δ_H 3.60 (1H, s) and δ_H 4.07 (1H, dt, J = 7.8 and 6.3 Hz) confirmed the presence of 17α- and 16β-hydroxyl groups, respectively.[6] The NMR data of **6** were consistent with the literature.[6]

Incubation of androstenedione **2** with *C. cladosporioides* MRC 70282 for 5 days yielded four metabolites (Table 1), which were identified by comparison of their ^{1}H and ^{13}C NMR spectra with those of **1** and with the previously isolated metabolites from the incubation of **1** with this organism.

Incubation of progesterone **3** with *C. cladosporioides* MRC 70282 for 5 days yielded two metabolites (Table 1). The first metabolite was identified as 5α-pregnane-3,20-dione **7**. The metabolite lacked the 4-H resonance of **3** at δ_H 5.73 (1 H, bs). The 19-methyl resonances of **3** at δ_H 1.18 ppm and δ_C 17.13 ppm revealed upfield shifts to δ_H 1.00 (Δ 0.18 ppm) and δ_C 11.43 (Δ 5.70 ppm), respectively. The ^{13}C NMR spectrum of **7** lacked the olefinic carbon signals of **3**. These results were in agreement with the reduction of the double bond in ring A. A comparison of the ^{13}C NMR spectrum of **7** with the literature showed that the reduction of the α,β-unsaturated system had taken place from the α-face, and consequently **7** was a 5α-H steroid.[8]

The second metabolite was identified as 21-hydroxypregn-4-ene-3,20-dione **8**. The ^{13}C NMR spectrum of **8** lacked the C-21 resonance of **3** at δ_C 31.29 ppm and showed a new resonance at δ_C 69.34 ppm, indicating the presence of a 21-hydroxyl group. The ^{1}H NMR spectrum of **8** lacked the 21-H resonance of **3** at δ_H 2.12 ppm and showed a new resonance (2H, bs) at δ_H 4.18 ppm, further indicating the presence of a 21-hydroxyl group. The NMR data of **8** were consistent with the literature.[9]

As can be seen from Table 1, *C. cladosporioides* MRC 70282 mainly hydroxylated testosterone **1** at C-16β, and this new hydroxyl group was then oxidised to a carbonyl group. A minor independent oxidation at C-17 and a minor epimerisation at C-17 were also observed. *C. cladosporioides* MRC 70282 reduced **2** at C-17, hydroxylated most of the substrate at C-16β and then oxidised almost half of it at C-16. A minor epimerisation at C-17 was also observed.

The hydroxylation of androst-4-en-3-ones at C-16β by *Aspergillus* niger,[5,6,10,11] *Whetzelinia* sclerotiorum,[6] *Corticium centrifugum*[12] and *Wojnowicia graminis*[13] has been previously reported. The biotransformation of testosterone **1** by *A. niger* KCH910,[5] *A. niger* ATCC 9142 (NRRL 599),[6,10] *W.* sclerotiorum,[6] *A. niger* TCCC 41650,[11] *C. centrifugum*[12] and *W. graminis*[13] gave some 16β-hydroxylated metabolites. 16β,17α-Dihydroxyandrost-4-ene-3-one **6**, which was isolated from the incubation of **1** with *C. cladosporioides* MRC 70282, had only been isolated from the incubation of **1** with *A. niger* ATCC 9142.[6] Incubation of androstenedione **2** with *A. niger* ATCC 9142 and *C. centrifugum* also gave some 16β-hydroxylated metabolites, although 16β,17α-dihydroxyandrost-4-ene-3-one **6** had not previously been isolated from these incubations.[10,12]

C. cladosporioides MRC 70282 mainly hydroxylated progesterone **3** at C-21, and this was accompanied by an independent 5α-reduction. *Aspergillus* brasiliensis,[14] *Rhizopus* nigricans,[15] *Cochliobolus spicifer* and a *Cladosporium cladosporioides* strain[16] had previously been reported to hydroxylate **3** at C-21. The *C. cladosporioides* strain mentioned also hydroxylated **3** at C-11 and C-17.[16] In this work, however, *C. cladosporioides* MRC 70282 only hydroxylated **3** at C-21 in a higher yield, and an independent minor 5α-reduction was also observed. 5α-Pregnane-3,20-dione **7** was also isolated previously from the incubations of **3** with *Penicillium* decumbens,[17] *Penicillium* digitatum,[18] *Ceratocystis* paradoxa,[8] *Aspergillus fumigatus*[19] and *Rhizopus nigricans*[20].

Although testosterone **1,** androstenedione **2** and progesterone **3** are all 4-ene-3-ketosteroids, testosterone **1** and androstenedione **2** lack the C-17 side chain of **3**. These results suggest that *C. cladosporioides* MRC 70282 might have metabolised **3** in a different way due to the presence of its C-17 side chain.

Experimental

Steroidal substrates were purchased from Sigma-Aldrich (Istanbul, Turkey). Solvents were of analytical grade and were purchased from Merck (Istanbul, Turkey). Potato dextrose agar (PDA) and agar for PDA slopes and ingredients for liquid medium were also purchased from Merck. The steroids were separated by column chromatography on silica gel 60 (Merck 107734) and eluted with increasing concentrations of ethyl acetate in *n*-hexane. Steroid mixtures from the column were then separated by column chromatography on aluminium oxide 90 active neutral (activity stage I, Merck 101077) using smaller columns for 2–3 hours. Thin-layer chromatography (TLC) was carried out with 0.2 mm-thick Merck Kieselgel 60 F_{254} TLC plates using ethyl acetate/*n*-hexane (1:1) as eluent. TLC plates were dipped in an anisaldehyde/H_2SO_4 reagent and heated to 120 °C for 3 min in order to visualise the spots. Infrared spectra were recorded using a Perkin Elmer Spectrum Two spectrometer. ^{1}H NMR spectra were recorded in deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS) as internal standard reference at 300 MHz on a Varian Mercury 300 spectrometer. ^{13}C NMR spectra were recorded in $CDCl_3$ at 75 MHz with a Varian Mercury 300 spectrometer. Chemical shifts are given in parts per million (ppm, δ scale) and coupling constants (J) are given in hertz (Hz). Melting points were determined with an Electrothermal IA 9200 melting point apparatus and are uncorrected.

C. cladosporioides MRC 70282 was obtained from TUBITAK, Marmara Research Center, Food Science and Technology Research Institute, Culture Collection Unit, Kocaeli, Turkey. Stock cultures were maintained at 4 °C on PDA slopes. Each biotransformation experiment was performed in duplicate and run with a control flask containing non-inoculated sterile medium and one of the substrates. After 5 days of incubation, each control was harvested and analysed by TLC. No metabolites were detected in the controls.

Biotransformation of testosterone **1**

The liquid medium for *C. cladosporioides* MRC 70282 was prepared by mixing glucose (20 g), peptone (5 g) and yeast extract (5 g) in distilled water (1 L).[21] The medium was evenly distributed among ten culture flasks of 250 mL capacity (100 mL in each) and autoclaved for 20 minutes at 121 °C. Spores, freshly obtained from a PDA slope,

Table 2 ^{13}C NMR data determined in $CDCl_3$ for compounds **1–8**

C atom	1	2	3	4	5	6	7	8
1	35.18	35.62	35.46	35.37	35.57	35.55	38.49	33.84
2	33.46	33.84	33.72	33.77	33.85	33.83	38.10	32.66
3	199.57	199.33	199.18	199.38	199.79	199.93	211.98	199.52
4	123.24	124.07	123.66	124.08	123.79	123.73	44.61	123.92
5	171.66	170.35	170.80	170.11	171.33	171.62	46.61	170.82
6	32.42	32.50	32.55	32.39	32.67	32.74	28.77	35.62
7	31.13	31.20	31.65	31.70	31.61	32.06	31.60	31.79
8	35.18	35.07	35.28	34.38	34.93	35.24	35.32	35.46
9	53.50	53.74	53.38	53.55	53.95	53.30	53.60	53.48
10	38.25	38.58	38.34	38.58	38.63	38.59	35.64	38.57
11	20.21	20.24	20.78	20.18	20.28	20.10	21.39	20.85
12	36.00	30.68	38.40	36.08	36.96	31.49	38.88	38.50
13	42.36	47.45	43.68	42.30	42.28	43.56	44.15	44.61
14	50.02	50.76	55.76	44.37	46.90	48.18	56.41	56.00
15	22.91	21.68	24.13	35.34	34.85	35.24	24.38	24.41
16	29.65	35.69	22.57	216.61	69.74	80.83	22.77	22.87
17	80.75	220.43	63.23	86.04	80.48	86.54	63.70	58.96
18	10.76	13.64	13.11	11.30	11.81	17.00	13.42	13.41
19	16.96	17.31	17.13	17.27	17.33	17.33	11.43	17.30
20			209.08	–	–	–	209.60	210.11
21			31.29	–	–	–	31.51	69.34

were transferred aseptically into each flask containing sterile medium in a biological safety cabinet. After cultivation at 28 °C for 3 days on a rotary shaker (160 rpm), testosterone **1** (1 g) dissolved in dimethylformamide (DMF) (10 mL) was evenly distributed aseptically among the flasks. The biotransformation of **1** was carried out in ten flasks for 5 days under these conditions. The fungal mycelium was separated from the broth by filtration under vacuum and the mycelium was rinsed with ethyl acetate (500 mL). The broth was extracted three times each with ethyl acetate (1 L). The organic extract was dried over anhydrous sodium sulfate and the solvent evaporated *in vacuo* to give a brown gum (1673 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in *n*-hexane yielded androstenedione **2** (79 mg, 8%), which was crystallised from acetone as prisms: m.p. 173–174 °C (lit.[6] 169–170 °C); IR (ν_{max}/cm^{-1}): 1736 and 1670; NMR: δ_H 0.91 (3H, s, 18-H), 1.21 (3H, s, 19-H), 5.75 (1H, s, 4-H); δ_C (Table 2).

Elution with 40% ethyl acetate in *n*-hexane yielded the unreacted starting material (205 mg), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Further elution with 40% ethyl acetate in *n*-hexane yielded 17β-hydroxyandrost-4-ene-3,16-dione **4** (325 mg, 31%), which was crystallised from ethyl acetate as prisms: m.p. 157–158 °C (lit.[10] 153–154 °C); IR (ν_{max}/cm^{-1}): 3460, 1755, 1670 and 1620; NMR: δ_H 0.80 (3H, s, 18-H), 1.24 (3H, s, 19-H), 3.77 (1H, bs, 17α-H), 5.75 (1H, s, 4-H); δ_C (Table 2).

Elution with 50% ethyl acetate in *n*-hexane yielded 16β,17β-dihydroxyandrost-4-ene-3-one **5** (211 mg, 20%), which was crystallised from acetone as needles: m.p. 180–181 °C (lit.[5] 177–179 °C); IR (ν_{max}/cm^{-1}): 3345 and 1670; NMR: δ_H 0.84 (3H, s, 18-H), 1.18 (3H, s, 19-H), 3.36 (1H, d, J = 7.5 Hz, 17α-H), 4.15 (1H, td, J = 7.5 and 5.0 Hz, 16α-H), 5.72 (1H, s, 4-H); δ_C (Table 2).

Further elution with 50% ethyl acetate in *n*-hexane yielded 16β,17α-dihydroxyandrost-4-ene-3-one **6** (22 mg, 2%), which was crystallised from acetone as plates: m.p. 175–176 °C (lit.[6] 171–174 °C); IR (ν_{max}/cm^{-1}): 3425, 2950 and 1650; NMR: δ_H 0.92 (3H, s, 18-H), 1.18 (3H, s, 19-H), 3.60 (1H, s, 17α-H), 4.07 (1H, dt, J = 7.8 and 6.3 Hz, 16α-*H)*, 5.71 (1H, s, 4-H); δ_C (Table 2).

Biotransformation of androstenedione **2**

Under the same conditions, the incubation of androstenedione **2** (1 g) with *C. cladosporioides* MRC 70282 for 5 days yielded a brown gum (1313 mg), which was then chromatographed on silica gel. Elution with 30% ethyl acetate in *n*-hexane yielded the unchanged starting material (177 mg), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Elution with 40% ethyl acetate in *n*-hexane yielded testosterone **1** (21 mg, 2%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Further elution with 40% ethyl acetate in *n*-hexane yielded 17β-hydroxyandrost-4-ene-3,16-dione **4** (317 mg, 30%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of the previously isolated metabolite.

Elution with 50% ethyl acetate in *n*-hexane yielded 16β,17β-dihydroxyandrost-4-ene-3-one **5** (256 mg, 24%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of the previously isolated metabolite.

Further elution with 50% ethyl acetate in *n*-hexane yielded 16β,17α-dihydroxyandrost-4-ene-3-one **6** (23 mg, 2%), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of the previously isolated metabolite.

Biotransformation of progesterone **3**

Under the same conditions, the incubation of progesterone **3** (1 g) with *C. cladosporioides* MRC 70282 for 5 days yielded a brown gum (1478 mg), which was then chromatographed on silica gel. Elution with 20% ethyl acetate in *n*-hexane yielded 5α-pregnane-3,20-dione **7** (121 mg, 12%), which was crystallised from ethyl acetate as needles: m.p. 197–198 °C (lit.[8] 198–201 °C); IR (ν_{max}/cm^{-1}): 1730 and 1710; NMR: δ_H 0.62 (3H, s, 18-H), 1.00 (3H, s, 19-H), 2.10 (3H, s, 21-H); δ_C (Table 2).

Elution with 30% ethyl acetate in *n*-hexane yielded the unreacted starting material (185 mg), which was identified by comparison of its ^{1}H and ^{13}C NMR spectra with those of an authentic sample.

Elution with 50% ethyl acetate in *n*-hexane yielded 21-hydroxypregn-4-ene-3,20-dione **8** (422 mg, 40%), which was crystallised from acetone as prisms: m.p. 144–145 °C (lit.[22] 137–140 °C); IR (ν_{max}/cm^{-1}): 3430, 1710, 1660 and 1620; NMR: δ_H 0.67 (3H, s, 18-H), 1.17 (3H, s, 19-H), 1.17 (3H, s, 19-H), 4.18 (2H, bs, 21-H), 5.72 (1H, s, 4-H); δ_C (Table 2).

Acknowledgement

We are grateful to Yavuz Derin for running the ^{1}H and ^{13}C NMR spectra.

Received 10 June 2018; accepted 22 July 2018
Paper 1805463
https://doi.org/10.3184/174751918X15337219180719
Published online: 13 August 2018

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